Chapter 4

Morphological and Biochemical Profiles of the Gonadal Cycle in the Sea Urchin *Paracentrotus lividus*: Wild Type vs. Bred

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Additional information is available at the end of the chapter

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Abstract

Paracentrotus lividus gonads represent a valued gourmet delicacy, particularly appreciated in Europe and in Japan. Their commercial value is generally associated to their size, freshness, colour and texture. Diet, gametogenesis and environmental conditions have a marked influence, promoting the indispensable mechanisms of synthesis, selective storage and mobilization of the bioactive compounds, as lipids, proteins and carbohydrates of gonads in order to obtain nutrients. The objective of this work is to compare the morphological and biochemical profiles of reproductive life cycle of the gonads of adult *P. lividus* in its marine natural environment and adult captured sea urchins breeding into a fish aquaculture system. The reproductive cycle of male and female wild and breeding *P. lividus* was characterized during 1 year by analysing variations of the gonadal content of lipids, proteins and carbohydrates of animals captured at four different locations of the south-western coast of Salento, Italy, with the animals grown in a fish farm and fed with four different types of diet. The gonadal and repletion indexes were determined before the specimen dissection for evaluation of sex, development stages and physiological aspects. Gonads were processed for histological and biochemical analysis. The gonadal content of lipids, proteins and carbohydrates was performed by the gas chromatography-mass spectrometry (GC-MS) and by spectrometry, respectively.

Keywords: sea urchin, gonadal cycle, lipids, proteins, carbohydrates

1. Introduction

The potential marketing of the edible sea urchin *Paracentrotus lividus* (Lamark 1816) has been considerably augmented during the last two decades due to its culinary value for various Mediterranean and mid-European populations. This fact poses particular interest for this invertebrate species [1] that is the most studied group of benthic macrofauna in the Mediterranean sea [4]. This edible Mediterranean sea urchin species has an extensive geographical distribution in Apulia sea, in the south of Italy [2]. However the local consumption of edible sea urchin is more substantial, coinciding with the peak of the tourist season. Local populations of sea urchins experience unavoidable pressure during that season, and in some cases, local authorities have imposed a temporary or permanent ban to protect them. Several studies have reported that sampling activities (fishing, harvesting) have direct consequences on the benthic macrofauna, potentially decreasing density and individual size and leading to sex ratio imbalance [3]. Furthermore, because edible sea urchins are frequently found in shallow waters, they are also subject to recreational fishing [4, 5].

The valuable food product of sea urchins is the gonads of female animals, referred as *roe*. The largest fisheries are in Japan, Chile and the USA, the latter two countries primarily exporting the product to markets in the Far East. The Chilean urchin fishery has increased dramatically in recent years to around 54,000 tons, largely due to the discovery of new fishing grounds. Smaller fisheries exist also in Europe, mainly supplying domestic markets. However the fishery statistics clearly demonstrate that most of the world's urchin fisheries are fully or overexploited, and it is generally accepted that further urchin fishing grounds are unlikely to be discovered. Whereas the biological basis for sea urchin culture has been long established, research continues to refine the hatchery production of the juveniles [6, 7]. To promote sea urchin production, different strategies, relatively to the optimal diets for sea urchin, in order to improve the quality of gonads (colour, taste and texture) are reported [8–11]. For example, in Japan the cages are allocated, immediately (juvenile period) in sea floor, but in other countries, the cages are first incubated in area with a low quantity of nutrient and later are arranged in sea floor [8–11]. Hatchery-reared juveniles have been grown in suspended culture with *Atlantic salmon*, *Salmo salar* [12–14]; in closed recirculation systems [15]; in land-based integrated systems [16] and in rock pools in southern Ireland (J. Chamberlain, Dunmanas Seafoods Ltd., pers. Comm.). Another interesting and cost-effective option is to obtain a uniform quality of gonads combining the advantages derived from the systems that produce a uniform size class, with the systems that use the coculture [17]. In north-west Scotland, *P. lividus* is being evaluated as a potential new species for aquaculture. *P. lividus* is often described as herbivorous, although there are documented instances of its feeding on artificial diets containing fish meal [18], sponges, hydrozoa, copepods, dead fishes and mussels [19]. Diet quality can significantly influence the sea urchins' somatic and gonadal growth [20–24].

Aquaculture has been supporting human demands for fish products for centuries and is an important industry worldwide [25]. Aquaculture fisheries are a booming industry but discharging heavy nutrient loads into coastal water [24] and on an intensive scale causing severe environmental problems. Modern intensive monoculture requires high inputs of water, feeds, fertilizers and chemicals and inevitably produces considerable wastes. Therefore, many aquaculture operations

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put enormous pressure on coastal habitats [26]. Waste products from fish farms consist mainly of nitrogen, phosphorus and carbon dioxide. A possible solution to this problem is to integrate seaweeds or filtrating species into fish farming. Numerous studies have been performed which combine seaweed culture with land-based fish tanks or open sea fish cages [24, 27–29]. Seaweeds removed up to 90% of the nutrients discharged from an intensive fish farm. Algal farming along the coasts, therefore, may function as an effective biofilter to alleviate the eutrophication problem worldwide [28–33]. Moreover, the use of filtrating species has also been considered in an integrated cocultured system and showed reasonably high efficiency in the removal of waste inorganic nutrients [24–29]. In this respect, the present research work is aimed to set up the best diet for the optimal breeding conditions of *P. lividus* in a coculture offshore fish farm. To this purpose the morphological and biochemical characteristics of sea urchin grown in their natural marine environment and in fish farm were compared. The wild samples were captured in four different sites along the Ionian sea of Apulia and near the offshore fish farm located in Torre Suda. The sampling sites were chosen: two, Capilungo and Posto Rosso, at south and two, Arcobaleno and Torre Pizzo, at north of fish farm (**Figure 1**). These animals were analysed during 1 year for the monitoring of the growth by comparing the population living in natural conditions, in relation to the sea flowing variability of available food with the population of sea urchins in breeding conditions and fed with different determined diets (**Figure 1**).

1.1. Gonad quality: the importance of lipids, protein and carbohydrates

Gonadal growth and maturation of sea urchins are characterized by an accumulation of nutrients in the nutritive phagocyte cells that are then used for gametogenesis. Pre-gametogenesis in both sexes begins with the gonad size increase by accumulation of nutrients into the phagocytic cells, filling the gonadal lumina. During gametogenesis, nutritive phagocytes gradually decrease in size, supplying nutrients to developing germ cells; lumina progressively are filled with eggs and sperms [34, 35]. Diet, gametogenesis and water temperature may have a marked influence on gonad quality, promoting the indispensable mechanisms of synthesis, selective storage and

Figure 1. (A) Map of Ionian Salento coast with the sampling sites of wild sea urchins. Location of fish farm is indicated by the circle; (B) overview of the cage distribution in the offshore aquaculture system at Torre Suda, Gallipoli, Italy.

contents [36–39]. Indeed, quality and quantity of food affect the reproductive maturation and growth of sea urchins [20–39] and biochemical composition of gonads [40–43]. Lipids, proteins and carbohydrates are all necessary for the sea urchin growth. Their presence in the gonads allows the correct gametogenesis.

Lipids, important energy reserves storing more energy per unit volume than proteins or carbohydrates, are structural components of cell and subcellular membranes and are vital for somatic growth. Lipids, like fatty acids (FA) and polyunsaturated fatty acids (PUFAs), are present in the gonads and are essential for a multitude of physiological functions. In addition PUFAs have also significant effects on human health, like eicosapentaenoic acid (EPA, C20:5 (n−3)) and docosahexaenoic acid (DHA, C22:6 (n−3)), that can prevent arrhythmia, cardiovascular diseases and cancer [44, 45]. A large group of FAs characterized by a furan ring (FFA) [46, 47], whose activity in nature is not completely clear, show a promising nutritional value, with scavenger activity against hydroxyl and peroxyl radicals [46]. The composition of these valuable FFAs, however, varies greatly among different urchin species and is influenced by their natural diet as well as physiological processes, that is, reproductive stage [18, 35]. FAs are also important for sea urchin reproduction. During gametogenesis, they can be used as a source of energy [48], and additionally, sea urchin spermatozoa obtain energy for swimming through oxidation of fatty acids derived either from phosphatidylcholine or from triglycerides [49]. The latter are important for larval development and survival [50].

Proteins are one of the most necessary and costly nutrients of most aquatic animal diets. Adequate provision of dietary protein decreases feed intake and increases growth and roe production in all species of sea urchin. On the other hand, high protein levels or the presence of specific amino acids could have an adverse effect on the quality of sea urchin roe. Therefore, an adequate protein diet should be formulated to get maximal growth and roe production, avoiding protein excess.

Soluble carbohydrates are easily digested by sea urchins, and numerous carbohydrates have been identified in the sea urchin gut [35], indicating that sea urchins can most likely utilize carbohydrates from a wide array of sources. Few studies have examined the relationship between dietary protein and dietary energy requirements in sea urchins. Understanding this relationship may be an important step in the formulation of a feed suitable for sustainable sea urchin aquaculture.

2. Materials and methods

2.1. Sample management

The study was carried out from July 2015 to June 2016 in a fish farm located 900 m far from the south coast of the Gallipoli (Lecce, Italy) area in the Ionic Sea (**Figure 1**). Four different cages are fed with artificial diet which was composed of a mixture in equal proportion of organic maize kernel (Zea mays), previously crushed with a blender into grains of a few millimetres, and chopped fresh organic spinach leaves (*Spinacia oleracea*); soya-based diet; *Ulva lactuca* fresh-based diet and pellet diet Classic K (Hendrix S.p.A). The diets were administered

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ad libitum; all cages are bred in coculture with *Sparus aurata* and *Dicentrarchus labrax*. The wild-type samples were collected monthly, in the same period, in four different localities: at south (Capilungo and Posto Rosso) and north (Arcobaleno and Torre Pizzo) of the fish farm. *P. lividus* are situated at 5–6 m depth. It is a steep zone and the bottom is mainly rocky. The diet of wild type was chiefly based on seaweed *U. lactuca*.

2.2. Biometric measures and histological studies

For each sampling site, 50 animals were collected randomly and brought back to the laboratory alive. Measurements of the "height" along the oral-aboral axis, in mm "diameter", perpendicular to the oral-aboral axis and "wet weight" in grams were taken for each animal. The gonadosomatic index (GSI) was calculated as GSI = 100 × wet weight of gonads/wet weight of whole animal. For the estimation of a feeding index, the gut contents of specimens per sample were dry-weighed and used to calculate a repletion index (RI) as proposed by Kempf [53], RI = 100 × wet weight of gut/wet weight of whole animal [51]. Specimens were then dissected to collect gonads that were weighted and the stage was determined. All the individuals with a gonad dry weight less than 4 g were considered to be immature or undifferentiated. Gonads were fixed in Bouin's liquid for histological studies. Samples were dehydrated in alcohol and embedded in agar-paraffin wax, and 2 μm sections were stained with hematoxylin and eosin [2]. Maturity of the gonads was estimated according to stages established by Lozano et al. [51] and Sanchez-Espana et al. [52], which included four phases of development in the female and two in the males.

2.3. Biochemical composition

2.3.1. Lipid extraction and quantification

Fatty acids were methylated with a modified version of the method proposed by Antongiovanni et al. [54]. The total lipids were extracted from fresh sample (gonads and gut) five times with hexane solution of internal standards, containing 1 mg/ml each of methyl valerate, methyl nonanoate, methyl tridecanoate and methyl nonadecanoate. Hence, 40 μl of methanolic KOH 2 N was added. The mixture was vortexed for 1 min at room temperature, and then the hexane phase was analysed by GC. The product of each extraction was filtered and then collected in one round glass flask.

Methylations were carried out in triplicate for each extract. Mixtures of fatty acid methyl esters (FAMEs) were analysed using a modified version of the method proposed by Santercole et al. [55], using one temperature programme with a 175°C plateau. Each FAME was identified using pure FAME reference materials, a custom reference mixture and Menhaden fish oil analytical standard (Sigma Aldrich, St. Louis, MO, USA). All chemicals and solvents were of analytical grade and were purchased from Sigma Aldrich (St. Louis, MO, USA). The sample was dried under vacuum by Rotavapor (Buchi) and the residue was dissolved with toluene. The transesterification reaction was performed with BF3 in methanol for 20 min at 90°C. Then by adding water and shaking vigorously, we obtained two phases, only the organic phase, containing lipids derivatized, was analysed by Gas Chromatografy with Flame Ionization Detector (GC-FID) for quantitative analysis.

2.4. Quantification of protein

To measure the protein content, 0.2 g of the gonad was homogenized with 3 ml of radioimmunoprecipitation assay buffer (RIPA buffer) and centrifuged at 3500 g for 10 min. Protein in the solution was measured by the Bradford method [56], with bovine serum albumin as a standard.

2.5. Quantification of polysaccharides

The polysaccharide content was extracted according to Unuma 2002. Polysaccharide in the solution was measured according to the anthrone-sulfuric acid method [57] with glucose as a standard.

3. Results

3.1. Gonadal growth and quality

The gonad maturity of female and male sea urchins was observed for 1 year in wild and breeding animals. Sea urchins have a reproductive cycle characterized by (i) a growing stage, the nutrients are accumulated in the nutritive phagocytes; (ii) a maturation stage, the nutrients are transferred to germ cells for gametogenesis and lastly (iii) a spawning stage, mature gametes are released from the gonad. The gonadal annual cycle for female distinguishes four stages: H1, H2, H3 and H4, while the gonadal cycle for male shows three stages M0, M1 and the spawning identified as M1s. In **Figure 2**, the histological analysis of gonadal cycle for female sea urchins is reported. In the first stage (H1), said *turned off* stage, ascini are devoid of gametes, the ascinal walls are thin and the lumen is filled with nutritive phagocytes. The primary oocytes are few along the ovary wall. The second stage (H2), or *oocytes maturation* phase, coincides with the onset of vitellogenesis and the consequent size increase of the primary oocytes. The oocytes remain attached to the ovarian capsule walls and are still surrounded by nutritive phagocytes. Primary oocytes of larger dimension start to migrate towards the middle of the ovary capsule and displace nutritive phagocytes. In the third stage (H3), which is the *spawning and post-spawning* stage, the ovaries are filled with eggs, and the nutritive phagocytes form a thin layer along the capsule ovary wall. The last stage (H4) consists in the gonadal reconstitution with a thick nutritive layer. In the capsule of the ovaries, the number of mature eggs is reduced, and empty spaces are observed due to post-spawning. In addition, the wall of the ovary is almost devoid of cells and is very thin. The histological analysis of female gonads from wild sea urchins grown in the different sites and those breeding did not show histological differences, while a temporal variability of gonadal cycle distribution during the year was observed (**Table 1**).

The stage M0, or *turned off* stage, of male gonadal cycle is characterized by lack of sperms and by nutritive phagocytes at the periphery. During the stage M1, the presence of sperm is observed, the mature testes are packed with spermatozoa and the nutritive phagocytes are absent or limited to the periphery. Males' specimens showed a similar pattern in all locations.

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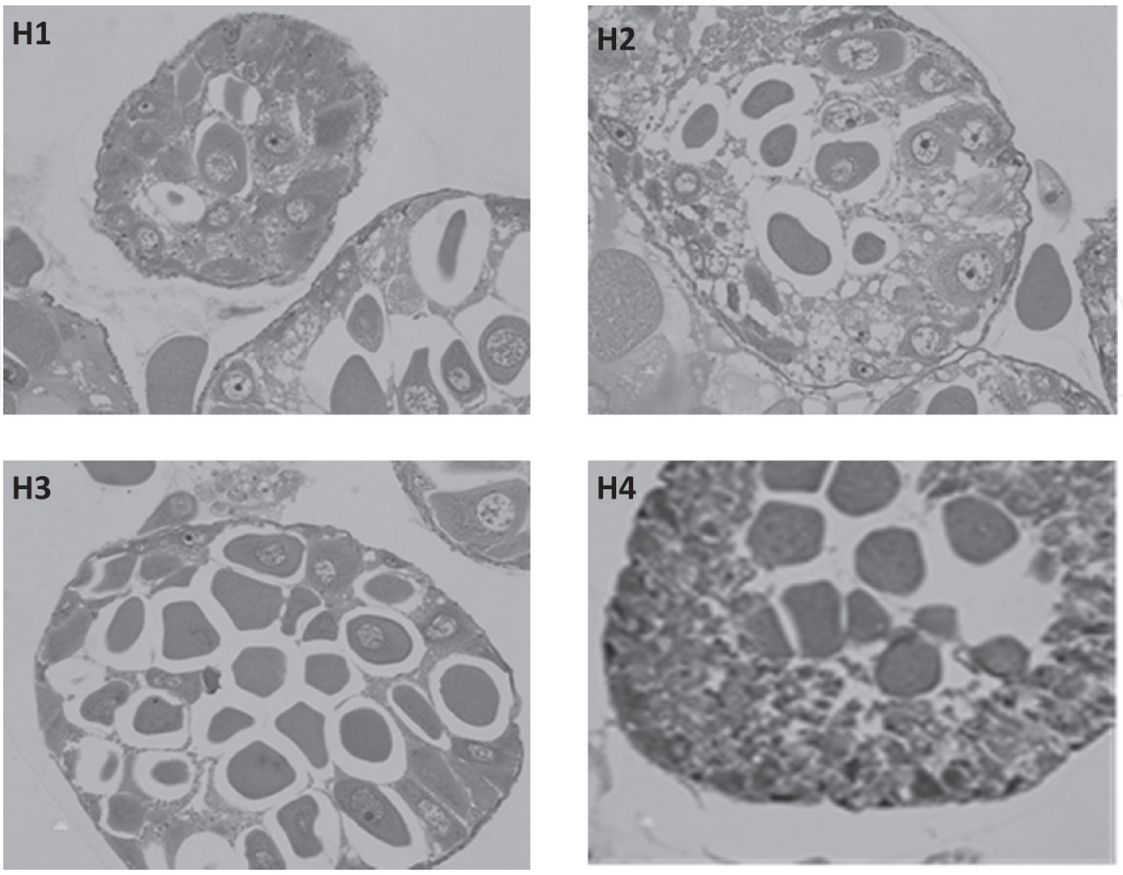

Figure 2. Histological representation of wild-type female gonad phases, that is, H1, H2, H3 and H4.

	H1	H2	H3	H4
Different sites				
Capilungo	Oct–Dec	Jan–Mar	Apr–Jul	Aug–Sept
Posto Rosso	Sep–Dec	Jan–Apr	May–Jul	Aug–Sept
Torre Pizzo	Sep–Nov	Dec–Feb	Mar–Jun	Jul–Aug
Arcobaleno	Sep–Nov	Dec–Feb	Mar–May	Jun–Aug
Breeding condition				
U. lactuca diet	Oct–Nov	Dec–Feb	Mar–Jun	Jul–Sep
Pellet-based diet	Sep–Dec	Jan–Mar	Apr–Jun	Jul–Aug
Soya-based diet	Sep–Dec	Jan–Apr	May–Jun	Jul–Aug
Artificial diet	Oct–Dec	Jan–Mar	Apr–May	Jul–Sept

Table 1. Annual distribution of gonadal cycle phases in female sea urchins.

The histological analysis of male gonads from wild sea urchins grown in the different sites and those in bred conditions shown a temporal variability of gonadal cycle distribution during the year (**Table 2**) (**Figure 3**).

	M0	M1	M1s
Different sites			
Capilungo	Oct–Mar	Apr–Jun	Jul–Sep
Posto Rosso	Sep–Mar	Apr–Jun	Jul–Aug
Torre Pizzo	Nov–Mar	Apr–Aug	Sep–Nov
Arcobaleno	Nov–Apr	May–Aug	Sep–Oct
Breeding condition			
Ulva lactuca diet	Oct–Feb	Mar–May	Jun–Sep
Pellet-based diet	Sep–Mar	Apr–Jul	Aug
Soya-based diet	Sep–Feb	Mar–May	Jun–Aug
Artificial diet	Nov–Apr	May–Jun	Jul–Oct

Table 2. Annual distribution of gonadal cycle phases in male sea urchins.

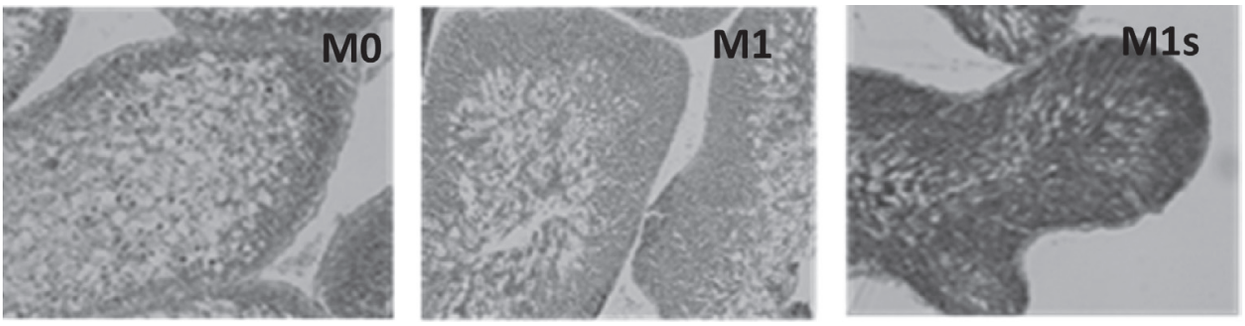

Figure 3. Histological representation of wild-type male gonads phases, that is, M0, M1 and M1s.

The gonadosomatic index (GSI) vs. size of wild animals captured at the four sites and of the animals fed with four different diets in the fish farm are shown in **Figure 4**. The lowest GSI values were observed in 15–35 mm size class collected in Capilungo, Posto Rosso and Arcobaleno, with Arcobaleno being the lowest one. In all four cages, the GSI of 15–35 mm size class of sea urchins are comparable to Torre Pizzo. A significant rise of GSI was observed in the 36–50 mm size class for all sites. High GSI values were measured for the 51–60 mm size class of Capilungo, while in Arcobaleno, the GSI value is comparable between 36–50 class and 51–60 class sizes. In Posto Rosso and Torre Pizzo, the GSI decreased significantly in the largest size class. In sea urchin cages, the GSI values of 36–50 mm size class were higher than for the wild conditions except for the animals fed with a soya-based diet.

The greatest repletion index (RI) values were found in the 51–60 mm size class in all four sites (**Figure 5**). The same trend was observed for the different sites: RI values increased with the increase of the size of sea urchins. Significant differences were found among the northern sites and southern sites: Capilungo and Posto Rosso measured the highest values for the 36–50 mm size class. The temporal trend of RI highlights the differences between the stations at north of Torre Suda (i.e. Capilungo and Posto Rosso) and those at south of Torre Suda (i.e.

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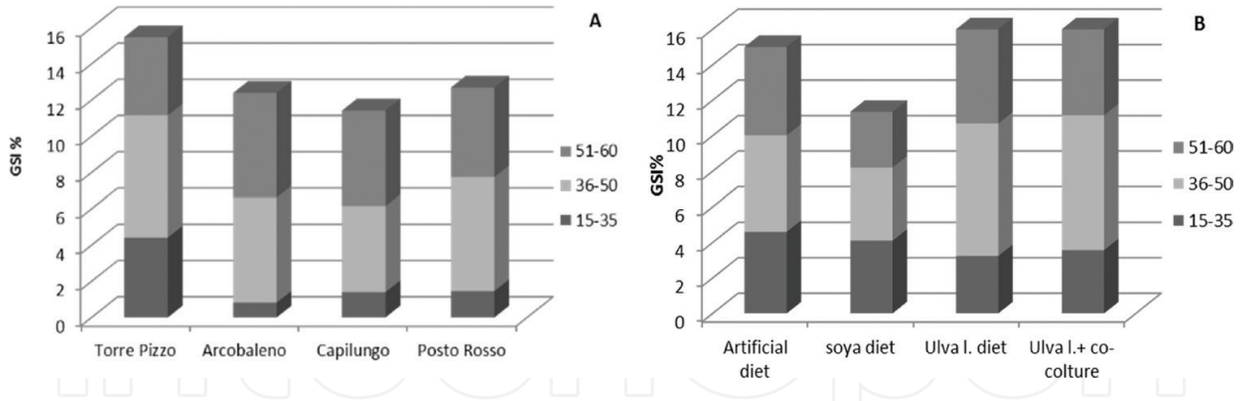

Figure 4. The gonadosomatic index (GSI) in wild and breeding animals.

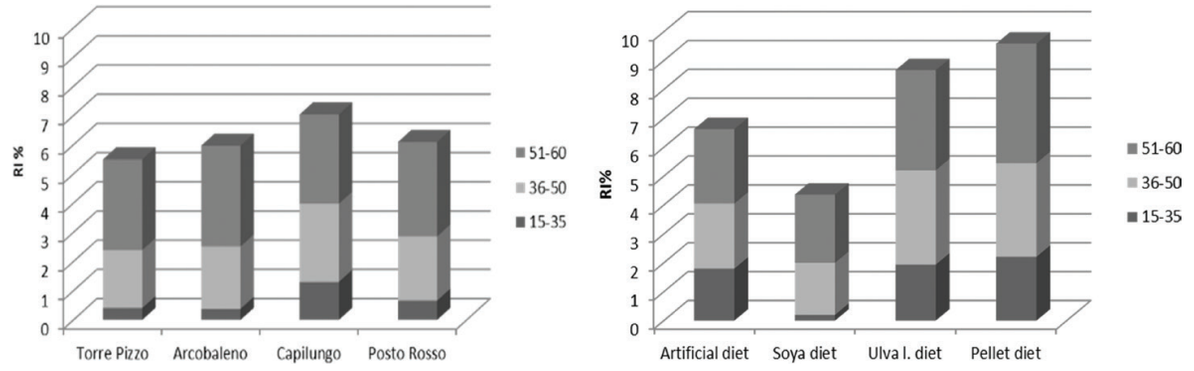

Figure 5. The repletion index (RI) in natural and in breeding conditions.

Arcobaleno and Torre Pizzo) in terms of values. In absolute, the highest RI values were measured in August in Capilungo and Posto Rosso, while the highest RI values for Arcobaleno and Torre Pizzo were measured in March. In the breeding sea urchins, the same trends of the environmental condition of Capilungo and Posto Rosso were observed; also in the breeding condition, the highest value was observed in August.

3.2. Biochemical profiles

Quantitative changes in the content of proteins, lipids and polysaccharides are analysed in wild samples (Capilungo, Posto Rosso, Torre Pizzo and Arcobaleno) and in breeding samples (four different diets). The content of proteins, lipids and polysaccharides decreased with the gametogenesis ongoing. The quantitative change of each class of molecules in the gonads during gametogenesis was standardized to the sizes of gonads. **Figures 4–7** show changes in the biochemical composition of ovaries and testes in function of phase of gonadal cycle (**Tables 1** and **2**) standardized to g100 g^{-1} body mass. Protein level in female gonads remained relatively constant between phases H1 and H2 (September and February) and decreased to a minimum value during phase H4 (July and August) in wild samples. Also in female gonads of breeding sea urchins, protein level remained relatively constant during the phases H1–H2 (October and March) and decreased to a minimum value during phase H4 (July and August).

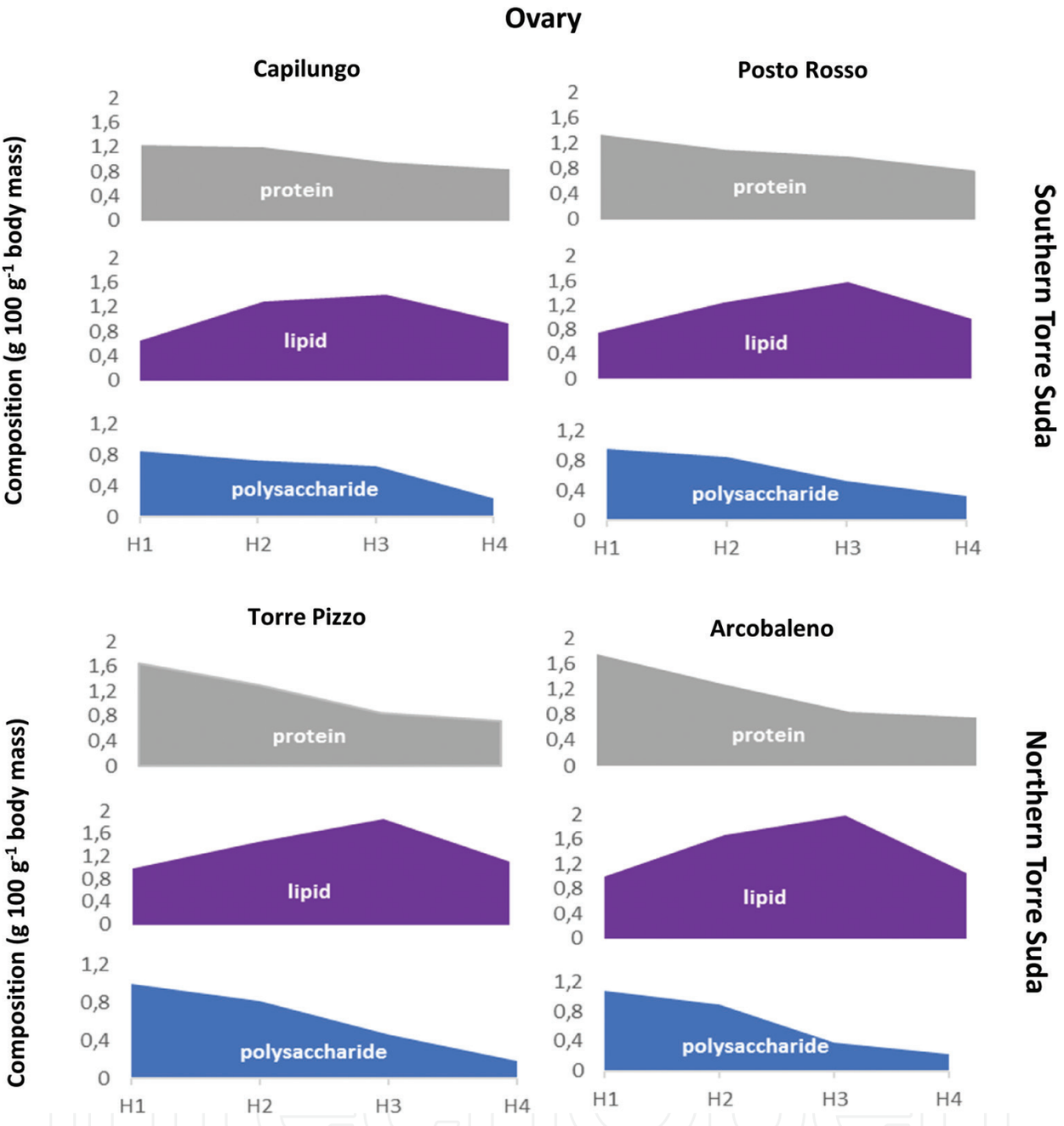

Figure 6. Biochemical composition of ovary of *P. lividus* wild type.

In wild male gonads, the content of protein is dependent on the site of sampling; in fact the maximum level of proteins in the wild samples grown at south of fish farm is registered during phase M1 (June); instead, the high value of proteins are registered in August at north of fish farm. In the breeding samples, the maximum content of proteins is recorded at the end of phase M1 (over spring/summer). The carbohydrate content remained constant in wild-type and breeding female gonads over phases H3 and H4, respectively (July and until June). The wild male gonads sampled at south of fish farm show a swift decrease in June, while the decrease was observed in August for the sample sites at north of fish farm. For the breeding samples, the speedy decrease of carbohydrate content is recorded during phase M1 (spring season). The

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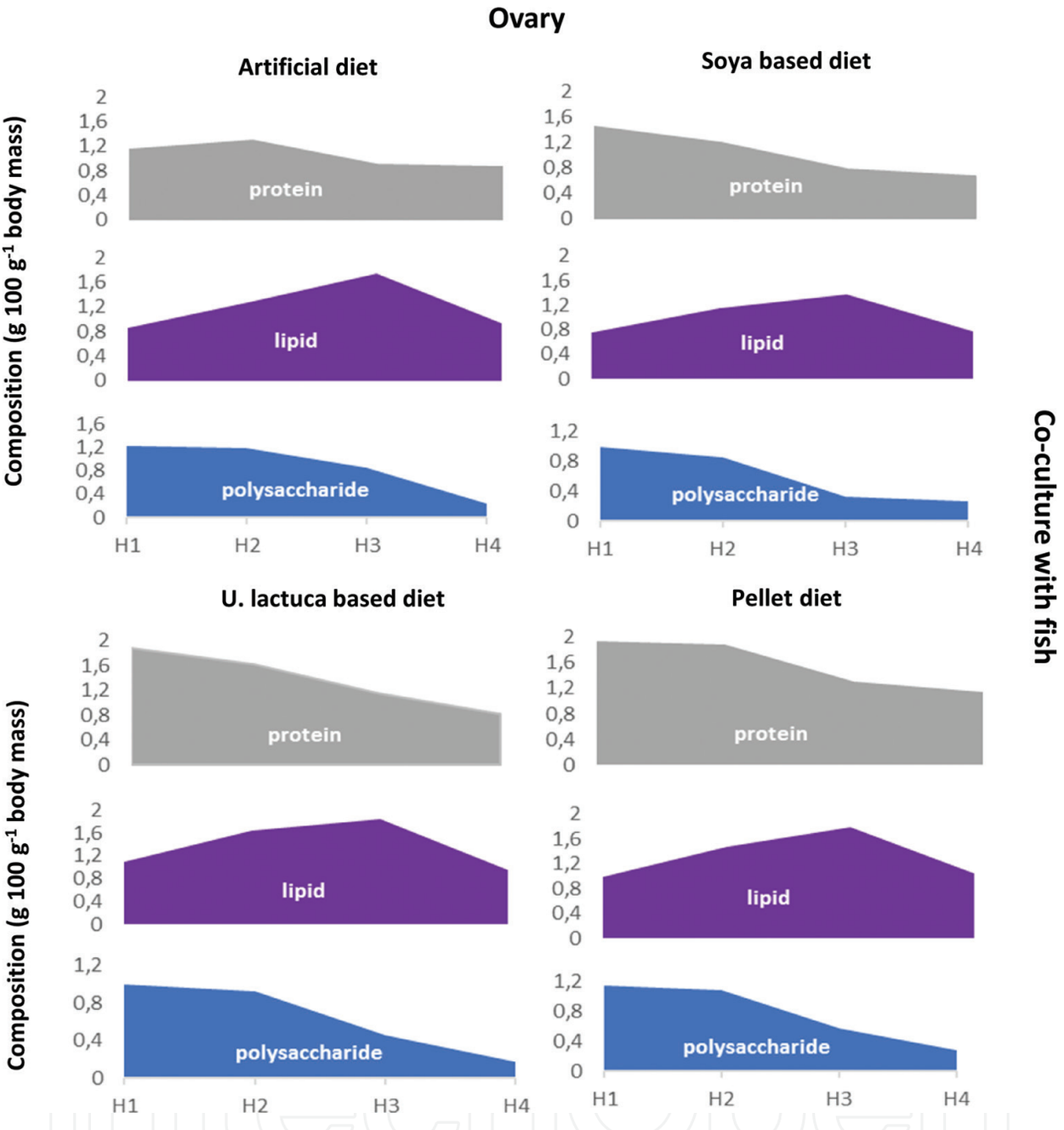

Figure 7. Biochemical composition of ovary of *P. lividus* bred.

lipid content underwent monthly variations in sea urchin gonads. In contrast to proteins and carbohydrates, the lipids are relatively growing until the start of phase H4 (July–August) in both wild and in bred female animals; afterwards the lipid content decreases simultaneously with the release of gametes. In the male gonads, the content of lipids was maximum during the phase M1 (spring season) for both wild and bred animals and decreased after the spawning.

In the light of these observations, it turns out that the process of gonadal maturation of breeding animals is retarded of about 1–2 months compared to the wild type. Moreover, there are also differences among the four groups of animals fed with different diets; the best content in

lipids, proteins and polysaccharides is found in cages fed with pellets and *U. lactuca*, followed by cages fed with artificial diet. Animals raised on a soy-based diet have a lower lipid profile than other breeding animals and wild. Artificial diet consumption was comparable to those recorded for the most preferred algae, whereas maize consumption was significantly higher than those referred to pellet. This feeding behaviour could be due to the high content of carbohydrates and proteins in maize with respect to algae and spinach which exhibit a very similar biochemical composition, characterized by a moderate content of protein and lipid intake (**Figures 8** and **9**).

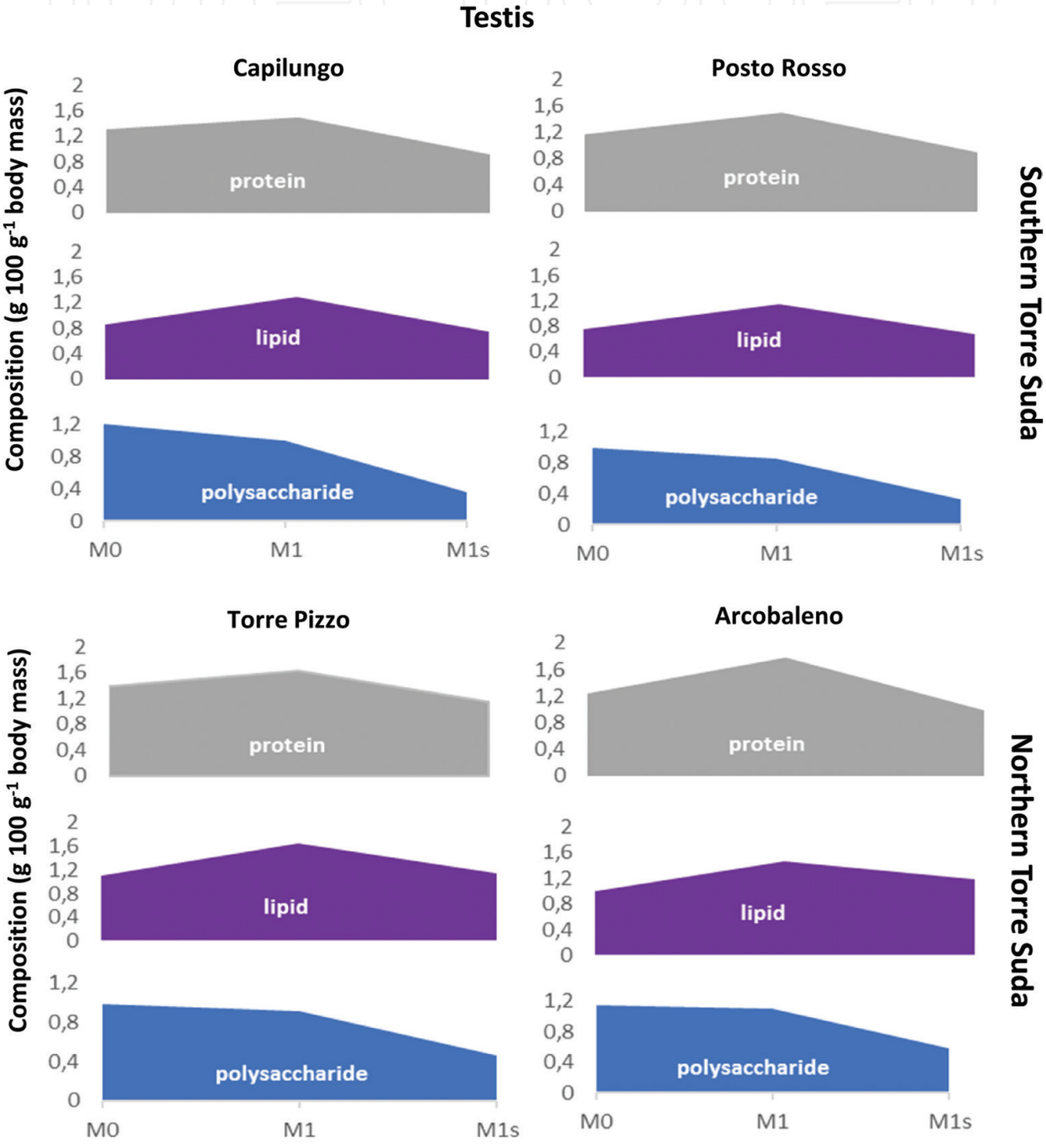

Figure 8. Biochemical composition of the testis of *P. lividus* wild type.

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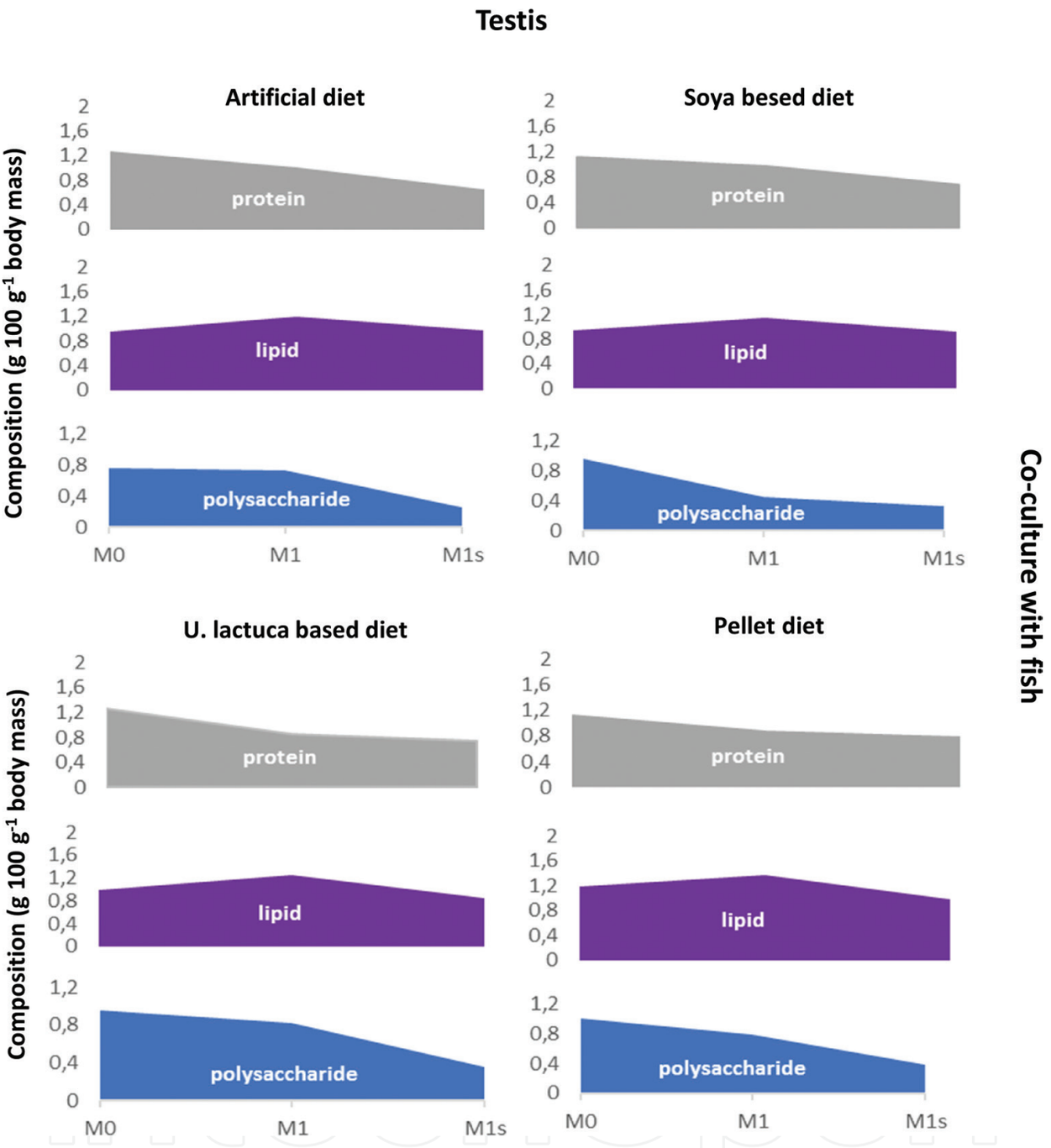

Figure 9. Biochemical composition of the testis of *P. lividus* bred.

4. Discussion

In the present work, the morphological and biochemical gonad characteristics of sea urchin grown in their natural marine environment and in cocultures with fish in an offshore fish farm were analysed for a period of 1 year. These data, which allows to define the best diet for sea urchins in view of the breeding set-up conditions, represent an important starting point for a scale production of sea urchins. Sea urchins are luxury food, and thus there is a growing economical interest

for their cultivation, but *P. lividus* is also central for its importance in determining the structure of rocky reefs [58]. Taking into consideration the fact that *P. lividus* is one of the most intensely harvested benthic invertebrate species for commercial and recreational purpose, the definition of optimal conditions to set up sea urchin culture is thus pivotal from broad perspectives. Our data demonstrated the good feasibility of a low-cost and easy-to-standardize diet, such as based on fresh *U. lactuca* and artificial in rearing vulnerable species, such as *P. lividus* in aquaculture system. The specimens of sea urchin demonstrated a good propensity to ingest both maize and spinaches, content in artificial diet, as it can be inferred from the RI reported in **Figure 5**; in addition, the GSI values related to this diet are the highest registered in this study. This diet also shows a low mortality rate, about 20%, compared to the other diets that bring a maximum value of mortality as 30% (data not shown). Other researchers have described the benefit of artificial diets over the use of maize: Repolho assessed the effect of captive brood-stock diet on fertilization and endotrophic larvae development of *P. lividus* obtained for maize diet [59, 60]. Histological analysis revealed a good quality of gonadal tissue in all cages, but compared to the wild animals, the gonadal cycle seems to be slowed down by 1–2 months, despite the biochemical profile of the gonads that does not reveal alterations in the amount of the different components. The biochemical composition of the sea urchin gonads has been studied in different species; the immature ovary and testis contained a big quantity of polysaccharides, lipids and proteins. In our study the polysaccharides decrease during the gametogenesis in both sexes and in wild and bred animals; according to literature these macromolecules are involved in the energetic metabolism [35, 48]. During the gametogenesis the lipids are stable for long time and decrease in the late phases of gonadal cycle for both sexes. Fatty acids, in fact, are an important energy source because they are needed by spermatozoa for swimming [49], in eggs for larval development and survival [50].

In conclusion, we propose an artificial diet to define a system offshore of sea urchin culture, in order to obtain gonads with comparable quality to that of wild type. This method permits to preserve the wild stocks and satisfy the market demand.

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